A ^1H -n.m.r. study of casein micelles

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The ^1H -n.m.r. spectrum of casein micelles consists of a small number of moderately sharp (linewidth approx. 60 Hz) resonances superimposed on the envelope of very broad lines expected for particles of this size. These sharp lines resemble, in chemical shift and relative intensity, the spectrum of the isolated 'macropeptide' released from the micelles by treatment with chymosin. The sharp lines in the casein micelle spectrum are further sharpened by addition of chymosin and broadened markedly by addition of ethanol. These observations are consistent with the proposal that the 'macropeptide' (the C-terminal 64 residues of κ -casein) forms flexible 'hairs' on the surface of the micelles.

Casein polypeptides account for more than 80% of the protein in bovine milk (Jenness, 1970). They exist predominantly in the form of micelles, which are very large and stable aggregates of the α_{s1} , α_{s2} , β and κ -casein polypeptides, together with deposits of calcium phosphate and citrate (Schmidt, 1980, 1982), removal of which causes disaggregation and disruption of the micelles. The overall diameter of the micelles ranges from 20 to 600 nm; they appear to be composed of 'submicelles' of diameter approx. 20 nm (M_r approx. 250 000) (Schmidt, 1980, 1982). While the detailed structure of the micelle is not known, the evidence suggests that much of the κ -casein is on the surface, and it has been suggested (Holt, 1975; Walstra, 1979) that segments of the κ -casein polypeptide protrude into solution, providing steric and/or electrostatic stabilization of the micelle against further aggregation. Removal of the polar, charged C-terminal segment of κ -casein (the so-called 'macropeptide') by the action of chymosin (EC 3.4.23.1) thus results in coagulation of the micelles such as takes place in the stomach during digestion of milk (cf. the preparation of junket). In the present paper we report ^1H -n.m.r. experiments designed to seek evidence for the existence of flexible segments of polypeptide chain in casein micelles.

Materials and methods

Materials

Chymosin (or rennin) was purchased from Sigma Chemical Co. All other chemicals were analytical grade.

Preparation of casein micelles

Raw Friesian cow's milk from a bulk storage tank was centrifuged for 20 min at 1000 $g_{av.}$ at 5°C, after which the cream was removed. NaN_3 was added to a final concentration of 0.02%. A portion (20 ml) of the skim milk obtained was filtered through a 1.2 μm Schleicher and Schüll filter. It was then centrifuged at 20 000 rev./min in a Beckman JA-20 rotor at 20°C for 2 h to spin down the casein micelles. The supernatant was discarded and the pellet of casein micelles was resuspended in 5 ml of simulated milk salts solution (Jenness & Koops, 1962) made up in $^2\text{H}_2\text{O}$ to $p^2\text{H}$ 6.2. This procedure was repeated several times in order to remove $^1\text{H}_2\text{O}$ and the whey proteins.

Treatment with chymosin and preparation of macropeptide

Casein micelles (2 ml, approx. 60 mg/ml) suspended in the $^2\text{H}_2\text{O}$ salts solution were treated with chymosin (0.002 mg/ml) at room temperature. The micelles coagulated within a few minutes. To separate the paracasein from the macropeptide the mixture was centrifuged as described above (after addition of an extra 1 ml of $^2\text{H}_2\text{O}$ salts solution, used to wash the mixture into the centrifuge tube). The supernatant containing the macropeptide was collected.

^1H -n.m.r. spectroscopy

Samples for n.m.r. spectroscopy consisted of casein micelles in $^2\text{H}_2\text{O}$ salts solution (60 mg/ml). Spectra were obtained at 270 MHz, with a Bruker

WH270 spectrometer. Between 1000 and 2500 free induction decays were averaged, using a 90° pulse and a pulse interval of 0.5 s. Before Fourier transformation, the free induction decays were multiplied by an exponential leading to a line broadening of 2–5 Hz; in some cases resolution enhancement by the convolution difference method (Campbell *et al.*, 1973) was used. The sample temperature was maintained at $25 (\pm 1)^\circ\text{C}$ by a stream of dry air. Chemical shifts are expressed

relative to 2,2-dimethyl-2-silapentane-5-sulphonate.

Results and discussion

The ^1H -n.m.r. spectrum of casein micelles is shown in Fig. 1(a). As would be expected for such large particles, the bulk of the intensity in the spectrum is in an envelope of overlapping, very broad lines. Of the order of 10% of the casein is

Fig. 1. 270 MHz ^1H -n.m.r. spectra of (a) casein micelles, (b) casein micelles (convolution difference spectrum), (c) casein micelles after addition of chymosin and (d) macropeptide isolated from chymosin-treated casein micelles. The vertical scales of these spectra are not identical.

present in milk as small aggregates of diameter $<40\text{ nm}$, the majority of which would have been lost during the centrifugation procedure used to transfer the casein micelles into the $^2\text{H}_2\text{O}$ salts solution (Schmidt *et al.*, 1973). The linewidths calculated for methylene or methyl protons rigidly attached to the remaining population of micelles range from 10 kHz for a 40 nm particle up to the rigid lattice limit of 80 kHz ; these are much too broad to be properly recorded with a high-resolution spectrometer.

However, it is clear that in the region of the spectrum containing resonances from aliphatic protons there are a number of moderately sharp lines. Overlap of these lines, together with the presence of the underlying broad signals, makes precise measurement of their linewidths impossible, but we estimate that these are between 50 and 70 Hz . These lines are thus two orders of magnitude narrower than would be expected for resonances from particles of the size of casein micelles. This clearly indicates that some segments of the polypeptide chains in the micelles have considerable flexibility (see Jardetzky & Roberts, 1981; Roberts *et al.*, 1983).

The sharp components of the spectrum can be seen more clearly in the spectrum in Fig. 1(b). This spectrum was obtained from that in Fig. 1(a) by using the convolution difference procedure of Campbell *et al.* (1973), with parameters chosen to remove the very broad signals while only slightly narrowing the sharp signals. It is clear that no sharp signals are observed in the region of the spectrum to low field of the water signal, where resonances from aromatic protons occur. The flexible segment(s) of polypeptide must therefore be very poor in aromatic amino-acid residues. From the methyl proton resonances observed in Fig. 1(b) these flexible regions do contain valine, leucine and/or isoleucine (resonance at 0.93 p.p.m.), threonine (1.22 p.p.m.) and alanine (1.43 p.p.m.) residues.

In view of the proposal (Holt, 1975; Walstra, 1979) that the C-terminal segment of κ -casein which is removed by chymosin may extend into the solution from the surface of the micelle, we examined the effects of chymosin on the ^1H -n.m.r. spectrum of casein micelles. This is shown in Fig. 1(c). The moderately sharp lines seen in Fig. 1(a) are now much more prominent, both because they are somewhat narrower and because the underlying broad signals have become much broader, so broad as to be virtually undetectable, due to coagulation of the micelles. The chemical shifts and relative intensities of these signals, however, remain essentially unchanged (cf. Figs. 1b and 1c). This is consistent with the idea that the peptide released into solution by chymosin (the macropep-

tide) is already protruding into the aqueous environment, where it has considerable mobility, in the native micelle before chymosin treatment.

The spectrum of the isolated 64-residue macropeptide is shown as Fig. 1(d). It is clear that this spectrum is very similar to Figs. 1(b) and 1(c). In particular the relative intensities of the macropeptide resonances at 0.93 p.p.m. (methyls of 7 isoleucine, 1 leucine and 6 valine residues), 1.22 p.p.m. (11 threonine residues), 1.39 p.p.m. (6 alanine residues) and 2.05 and 2.28 p.p.m. (predominantly 10 glutamate and glutamine residues, and 8 proline residues) are very similar to the relative intensities of the corresponding resonances in Fig. 1(b), indicating that the amino acid composition of the flexible segment of polypeptide is similar to that of the macropeptide. (In addition, neither has any aromatic residues.) The lines in Fig. 1(c) are rather broader than in Fig. 1(d), presumably due to 'trapping' of the macropeptide in the coagulum. However, the resonance from the citrate in the buffer (a quartet centred at 2.63 p.p.m.) is also much broader in the presence of the casein micelles than in their absence. This may be partly due to local magnetic susceptibility differences produced by the micelles, but it may also reflect exchange of the citrate in the buffer with part of the citrate that is bound to the micelles (Pyne & McGann, 1960).

There is hydrodynamic evidence supporting the idea that the C-terminal segment of κ -casein extends into solution from the surface of the micelles. The hydrodynamic radii of casein micelles are found to be significantly greater than those estimated by electron microscopy (Schmidt *et al.*, 1974; Griffin & Anderson, 1983), suggesting the existence of a surface layer of flexible protein segments which entrains significant amounts of solvent water. If these flexible segments consist primarily of the C-terminal part of κ -casein, which is strongly negatively charged (8 negatively and 0 positively charged residues out of the last 51; Mercier *et al.*, 1973), then treatment with chymosin should result in a decrease in the hydrodynamic size of the micelles before the onset of coagulation. This has in fact been observed by using dynamic light scattering measurements (Walstra *et al.*, 1981; Horne, 1984; M. C. A. Griffin, unpublished work).

Further light-scattering experiments by Horne (1984) showed that addition of ethanol (up to 20% , v/v) caused a progressive decrease in the hydrodynamic size of the micelles. The maximum decrease in hydrodynamic diameter was approximately equal to the initial decrease in size caused by the action of chymosin in the absence of ethanol. Furthermore, in the presence of ethanol (18% , v/v) there was no initial drop in hydrodyna-

mic size on treatment with chymosin. This was interpreted as shrinkage or retraction of the 'hairs' against the micelles caused by ethanol. We have observed that addition of ($^2\text{H}_6$)ethanol to casein micelles leads to progressive broadening of the sharp component of the ^1H -n.m.r. spectrum until at 18% (v/v) ethanol it has virtually disappeared (results not shown). Together with the observation of the effects of chymosin, this supports the idea that the parts of the protein responsible for the anomalous hydrodynamic behaviour of the micelles are the same as those giving rise to the sharp ^1H -n.m.r. signals.

Both the hydrodynamic and the ^1H -n.m.r. experiments are thus entirely consistent with the proposal (Holt, 1975; Walstra, 1979) that the C-terminal segment of κ -casein (the macropeptide) is flexible and extends into solution from the surface of the micelle.

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